Studies on Nuclear Transfer Techniques for Generation of Transgenic-cloned Calves and Goat

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ABSTRACT

The purpose of this study was to evaluate the feasibility of using somatic cell nuclear transfer (SCNT) technique for producing the clone transgenic bovine. Through the international cooperation between Professors Jerry Yang at University of Connecticut (UCONN) and Winston T. K. Cheng at National Taiwan University (NTU), the enucleation rate and fusion rate of nuclear transferred (NT) embryos were 89% (1170/1311) and 88% (938/1064), respectively. Moreover, the development rates of the NT-embryos cloned from adult ear fibroblast cells of which had been transfected with human blood clotting factor VIII (*aLA-hFVIII*) gene were 90% (670/741), 59% (384/741), and 38% (278/741) reached 2- ~ 8-cells, 16-cells and blastocyst stage, respectively, after having been cultured for 7 days *in vitro*. One hundred and ninety-three bovine cloned transgenic embryos were transferred into 82 recipient females. Eleven of them were pregnant, with only 5 giving birth to 5 cloned calves. Unfortunately, one of the five cloned calves was dead after birth. While none of those four survived cloned calves was evidenced harboring with the *aLA-hFVIII* transgene when their genomic DNA had been subjected to the southern blot analysis, respectively, one cloned dairy goats carrying the *aLA-hFVIII* transgene has been successfully generated based on the same strategy described above. In conclusion, this study has successfully produced five cloned calves and one cloned-transgenic goat by SCNT technique.

Key words: Bovine, Nuclear transfer, Fibroblast cell, Transgenic, *Human blood clotting factor VIII (aLA-hFVIII)*.

INTRODUCTION

Somatic cell nuclear transfer (SCNT) method has been used to clone several mammalian species including sheep (Wilmut *et al.*, 1997), cattle (Kato *et al.*, 1998), goats (Baguisi *et al.*, 1999), pigs (Polejaeva *et al.*, 2000), rabbits (Chesne *et al.*, 2002), mice (Wakayama *et al.*, 1999), horses (Galli *et al.*, 2003), mules (Woods *et al.*, 2003), and cats (Shin *et al.*, 2002). Several adult somatic cell types, including mammary gland cells (Wilmut *et al.*, 1997), oviduct epithelial cells (Kato *et al.*, 1998), cumulus cells (Wakayama *et al.*, 1998), mural granulosa cells (Wells *et al.*,

1999), muscle cells (Shiga *et al.*, 1999), uterine epithelial cells (Cho *et al.*, 2000), and ear fibroblasts (Kubota *et al.*, 2000) were used to produce these clones.

In Taiwan, both transgenic (Tg) pigs and Tg dairy goats harboring human clotting factor IX and factor VIII cDNA(s) have been generated by direct microinjection of foreign DNA into the pronucleus of zygotes, respectively, the overall efficiency of this process is only around 1%. The technique of SCNT in mammals has been developed to produce cloned and transgenic animals. Moreover, the technique of SCNT has been proven more successful in generating transgenic animals than that pronuclear microinjection (Schnieke *et al.*, 1997). The advantage of using NT to produce transgenic animals is the ability to use preselected genetically modified cells as donor nuclei (Schnieke *et al.*, 1997).

Therefore, in this study, we evaluated the feasibility of using SCNT technique to produced cloned-transgenic livestock. We have successfully produced five cloned calves and one cloned-transgenic goat so far.

MATERIALS AND METHODS

1. Oocyte collection and enucleation

(1) Oocytes collection in goat

In vivo matured caprine oocytes were used for nuclear transfer. Estrus of donor does were synchronized by intravaginal releasing device with 366 mg of progesterone (CIDR®, AHI Inc. New Zealand). Each animal was injected with 125 μg of the $PGF_2\alpha$ analogue (cloprostenol, Estrumate®, Coopers, Germany) on day 11. For superovulation, animals were injected with a total of 18 units of pFSH (Tenka Pharmacy Co. Japan) plus a total of 11.2 units of LH (Sigma L-5269), which were divided into 6 injections for three days 24 h before removal of the CIDR. Single dose of 1500 IU of hCG (Gona-500®, China Chemical & Pharmacy Co. Taiwan) was administered 8 h after the last FSH injection. The surgery of the oocyte donors was performed 22 h after hCG administration. The cumulus-oocyte complexes (COCs) were recovered by flushing the uterine horns with Dubelco's phosphate-buffered solution (PBS) using an 18G plastic catheter. Collection of the COCs was performed from the cranial part of the uterine horn based on the protocol described by Shen (2003). The COCs had their cumulus cells removed by gentle pipetting in the hyluronidase (Sigma H-3506) solution (1 mg/ml in PBS) for 3 min. Only the oocytes with first polar body (PB1) were used for enucleation.

(2) Oocytes collection in cattle

The cattle ovaries were taken from slaughterhouse. After ovaries collected, the COCs were collected from follicles (2-8 mm diameter) on the surfaces of the ovaries by a 1 ml syringe with No. 18 needle under negative pressure. The quality well and cumulus cells completed oocytes were chosen for *in vitro* maturation. According to the bovine oocyte maturation step described by Shen (2003), ten to twenty of the COCs were transferred in the maturation medium (5% FCS M199) and cultured in the 38.5°C, 2% CO_2, 98% air and saturated humidity condition for 18-19 hours. The externally matured oocytes containing the first polar body were chosen for enucleation.

(3) Enucleation of goat and cattle oocytes

The enucleation of goat and cattle oocytes were achieved by cutting the zona pellucida and pushing out the PB1 and its surrounding cytoplasm with a glass needle. Successful enucleation was confirmed by

Hoechst 33342 staining of the remaining ooplasm (Kubota *et al.*, 1998).

2. Collection and culture of ear fibroblast cells

Skin biopsies were obtained from the ears of a 2-year-old Alpine doe and a 2-year-old Holstein cow of which with high milk yield. The ear tissues were cut into small pieces (3 mm^2) and then cultured as tissue explants in cell culture medium at 38.5 ℃ in a humidified incubator containing 5% CO_2 in air. When fibroblast cells had formed monolayers surrounding the tissue explants after 10 days culturing, the explants were then removed and the fibroblast cells cultured to confluency. The cell strain was routinely maintained on dishes until passage 10 and then stored frozen (Kubota *et al.*, 2000).

3. Transfection of foreign gene into ear muscle fibroblast cells:

A plasmid contained the full-length cDNA sequences of human coagulation factor Ⅷ (hF Ⅷ) and the neomycin resistance gene driven by the bovine α-lactabumin (αLA) promoter and the simian virus-40 (SV-40) promoter, respectively, was linearized and delivered into the ear skin fibroblast cells using electroporation method (150 V, 10 msec, 2 pulses). The transfected cells were suspended in the medium contained DMEM supplemented with 10% FBS and subjected to *in vitro* culture at 37 ℃ in a humidified atmosphere of 5% CO_2. Positive cells harboring the transgene(s) were selected for a period of 20 days using this same medium containing 1 mg/mL neomycin as the method described by Schnieke *et al.* (1997). Selected cell lines were frozen into small aliquots for later use in nuclear transfer studies.

4. Preparation of donor cells

Donor cells harboring the αLA-hF Ⅷ gene, after thawing and plating into 4-well plates and culture in DMEM plus 10% FBS and 1% penicillin/streptomycin till confluent, had been subjected to serum starvation (0.5% FBS in DMEM) for 5~8 days before they were collected by trypsinization, and then resuspended in DMEM containing 0.5% FBS for nuclear transfer (Kubota *et al.*, 2000). Both enucleation of reciepient oocytes and transfer of donor cells were manipulated within a drop of the handling medium (PBS containing 10% FBS). For nuclear transfer, each of the donor cells, sized less then 20 μm with smooth plasma membrane, was picked up with a manipulation pipette and slipped into perivitelline space of the enucleated oocyte. Immediately after cell transfer, cell-cytoplast couplets were subjected to electrical fusion within mannitol fusion medium (0.32 M mannitol + 100 μM $CaCl_2$ + 100 μM $MgSO_4$ + 0.01 mg/ml BSA (sigma A-8806)) according the procedures described by Kubota *et al.* (1998). Briefly, each of the manipulated embryo was aligned by a pair of electrodes so that the contact surface between the donor cell and the enucleated oocyte was parallel to the electrode surface and a single pulse with 10 μsec in pulse length at 1.34 kV/cm electrical field strength was given by using the BTX Electrocell Manipulator 2001. After exposure to the fusion pulse, the fused couplets were placed and incubated in drop of 80 μl TCM-199 plus 5% FBS and 1 μl/10 ml gentamycin under mineral oil incubated at 38.5℃ in 2% CO_2 till activation (Shen *et al.*, 2003).

5. Cell insertion and electrofusion

The enucleated oocytes and dispersed donor cells were manipulated in PBS containing 10% FBS. The donor cell (<20 μ

m) with smooth plasma membrane was picked up with a manipulation pipette and slipped into the perivitelline space of the enucleated oocyte. Before fusion, a cell-cytoplast couplet was aligned and gently pressed by a pair of electrodes in the fusion medium [0.32 M mannitol + 100 μM $CaCl_2$ + 100 μM $MgSO_4$ + 0.01 mg/ml BSA (sigma A-8806)] (Kubota *et al.*, 2000). Thereafter, a brief electrical pulse (15 μ sec) at 2.33 kV/cm was administered using a BTX Electrocell Manipulator 2001. After the EP, the fused couplets were incubated in 5% FBS M199 medium covered with mineral oil (sigma M-8410) at 38.5℃ in 2% CO_2 till activation (Shen, 2003).

6. Activation and culture of the reconstructed embryos

After 4-5 h electrical fusion, the reconstructed embryos were activated by 5 μM calcium ionorphore (A23187) for 5 min and then immediately cultured in 2 mM 6-dimethylaminopurine (6-DMAP) for 4 h. The activated NT-embryos were then cultured in 5% FBS M199 medium at 38.5℃ in 2% CO_2 (Shen, 2003).

7. Embryo transfer

In goats, estrus synchronization of recipient doe was induced following a protocol similar to donors' doe except with 500 IU eCG (Sero-Gona®, China Chemical & Pharmacy Co., Taiwan) treatment on day 11 conjuncted with the $PGF_2\alpha$ analogue. After mid-ventral incision, the uterus was exteriorized and 1-2 cell stages embryos transferred into the oviduct ipsilateral to the ovary with more ovulation points.

In cattles, the recipient cows were derived from Holstein in natural estrus. Embryos that reached the blastocyst stage were transferred into recipient cattle approximately 7 days after synchronized estrus. One or two embryos per recipient were nonsurgically introduced into the uterine horn ipsilateral to the ovary containing a palpable corpus luteum.

Pregnancy evaluation of recipient does was performed using transrectal ultrasound at 45 days after embryo transfer, and recipient cows at 53 days (Shen, 2003).

RESULTS AND DISCUSSION

The SCNT technique in Taiwan has been successfully established since the last 3 years after the co-operation between NTU and UCONN. Results in cattle cloning showed, the enucleation rate and fusion rate of bovine NT embryos were 89% (1170/1311) and 88% (938/1064), respectively. The development rates of the NT-embryos cloned from adult ear fibroblast cells of which had been transfected with human blood clotting factor VIII (*aLA-hFVIII*) gene were 90% (670/741), 59% (384/741), and 38% (278/741) reached 2-cells, 16-cells and blastocyst stage, respectively, after cultured for 7 days *in vitro* (Table 1). One hundred and ninety-three bovine cloned transgenic embryos were transferred into 82 recipient females. Eleven of them were pregnant, with 5 giving birth to 5 cloned calves (Table 2). Unfortunately, one of the five cloned calves was dead after birth. However, none of those five survived cloned calves was evidenced harboring with the *aLA-hFVIII* transgene with southern blot analysis.

The fusion and cleavage rates of goat NT-embryos cloned from adult ear fibroblast cells with *hFVIII* gene transfected were 93% (100/107) and 23% (23/99), respectively (Table 3). One out of 11 recipients were pregnant and gave birth to 1 kid (1.1% of cloned embryos transferred) after transferring 92 NT-embryos (Table 4).

Table 1. The in vitro development of bovine NT-embryos cloned with *hFVIII* gene transfected adult ear fibroblast cells.

Enucleation Rate (enucleated/ examined)	Fusion Rate (fused/ examined)	No. of cultured NT-embryos	No.(%) of NT-embryos developed to*			
			2-cell	16-cell	CM	B
89.2 1170/1311)	87.5 (938/1064)	741	670(90.4)	384(58.5)	337(45.5)	278(37.5)

* CM: Compacted morula; B: Blastocyst.

Table 2. The results of embryo transfer with bovine NT-embryos cloned from an adult Holstein ear fibroblast cells of which transfected with *hFVIII* gene.

No of NT-embryos transferred	No. of recipients	No. (%) of recipients became pregnant	No. (%) of offspring
193	82	11 (13.4)	5 (6.14)

Table 3. The cleavage rate of goat NT-embryos cloned with *hFVIII* gene transfected adult ear fibroblast cells.

No. of NT-couplets	No. (%) of NT-couplets Fused	No.(%)of NT-embryos cultured	No. (%) of NT-embryos developed to 2-cell after culture
107	100(93)	99	23(23)

Table 4. The results of embryo transfer with goat NT-embryos cloned from an adult Alpine ear fibroblast cells of which transfected with *hFVIII* gene.

No of NT-embryos transferred	No. of recipients	No. (%) of Recipients Became pregnant	No. (%) of offspring
92	11	1 (9.1)	1 (9.1)

Moreover, the kid was evidenced harboring with the *aLA-hFVIII* transgene when their genomic DNA had been subjected to the southern blot analysis.

In this study, the cloning efficiency was evaluated based on the number of cloned offsprings produced over the number of embryos transferred. The overall cloning efficiency in bovine and goat were 2.6% (5/193) and 1.1% (1/92), respectively. It suggested that the cloning efficiency of bovine and goat were still low in Taiwan. It is known that the cloning efficiency is closely associated with epigenetic reprogramming (Kang *et al.*, 2001; Xue *et al.*, 2002). Despite the recent successes in cloning various animal species, using somatic cells as the source of donor nuclei has raised many practically relevant problems such as increasing abortion rates, high birth weight

and perinatal death (Campbell *et al.*, 1996; Wilmut *et al.*, 1997; Schnieke *et al.*, 1997). The production of viable offspring derived from NT depends on the successful combination of numerous factors including the quality and age of recipient oocytes, the procedures for reconstruction of the NT embryos, the culture conditions, as well as the cell cycle stages of both the donor and recipient cells (Campbell, 1999).

Calves and lambs resulting from embryos produced in vitro may be heavier at birth weight than that produced from *in vivo* (Willadsen *et al.*, 1991; Keefer *et al.*, 1994; Behboodi *et al.*, 1995; Sinclair *et al.*, 1995; Walker *et al.*, 1996) and increased incidences of neonatal mortality (Schmidt *et al.*, 1996; Kruip and den Daas, 1997). These phenomena might be attributable to the suboptimal culture conditions such as serum in the medium or the manipulation procedure itself (Garry *et al.*, 1996; Kruip and den Daas, 1997). In this study, reconstructed goat embryos transferred into the oviducts of the recipients during 1- to 2-celled stages, but the bovine NT embryos were transferred during blastocyst stage after *in vitro* culture for 7 days. Therefore, the goat NT embryos only exposed to a relatively short period of *in vitro* culture compared to bovine NT embryos. This may have explained why the cloned kid healthy grew, but one of five cloned calves was died after birth. On the other hand, the post-neonatal death occurred in our cloned calf might due to those processes for adopting to the nuclear transfer adopted. The major problem might be resulted from the quality of those recipient oocytes employed. The fact of using poor quality of oocytes as the recipient cytoplasts might have impairments of both developmental capacity and gene expression in those embryos reconstituted after nuclear transfer. Also, some inappropriate epigenetic-reprogramming events might have therefore been resulted (Kang *et al.*, 2001). Unfortunately, the ovaries obtained from abattoirs in Taiwan were usually provided from the cows with infertility syndrome which might have consequently resulted in poor quality of oocytes essentially used in our previous studies.

Whether the pregnancy rate after the embryo transfer is consistent with the production rate of cloned calf remains to be verified. After all, the number of genes to be turned on during the single cell's blastocyst stage is much less than the number of cells needed for a grown-up. In the development process of cloned embryo, the bottleneck facts on both the high fetus loss after pregnancy and the low postnatal survivability after birth are much tougher than the development ability before the preimplantation of embryos (Heyman *et al.*, 2002). Therefore, the cow embryos cloned in Taiwan with a satisfactory development to blastocyst stage in vitro may not necessarily mean that they possess the same developmental potentiality to culminate normal offspring born after re-implantation to the recipients. Using the *ovum pick up technique* to collect the *in vivo* maturation of bovine oocytes as recipient cell might be able to solve the problem partially. However, there is no explanation for the relationships between the cloned died calf and the inferior quality of recipient oocyte.

A number of related problems such as the early losses of somatic cell cloned embryos, fetus losses, larger offspring syndrome, inferior adaptation, and postnatal mortality has been studied (Campbell *et al.*, 1996; Wilmut *et al.*, 1997; Schnieke *et al.*, 1997; Wells *et al.*, 1999; Cibelli *et al.*, 1998). Some researchers believe that these issues were resulted from nuclear transfer process, reprogramming of donor nuclei, varied donor cells and *in vitro* culture system as Wells *et al.* (1998) mentioned in their report. As far as the nuclear transfer animals concerned, the abnormal situations developed by the maternal placental are often the main cause for the embryos early losses (Stice *et al.*, 1996); and the results in hydrallantois, enlarge placenta and umbilicus, and placental abnormality in the later gestation (Kruip and den Daas, 1997). As a result, the probability of neonatal is approximately one fourth of the *in vitro* produced embryos of *in vitro* maturation and *in vitro* fertilization (Wells *et al.*, 1998). Heyman *et al.* (2002) found that the abortion and the prthologic placentation of cloned calves were probably highly related to the pregnancy serum protein 60 (PSP60) secreted by the recipient. They indicated that the excessive PSP60 found in the blood of recipient female of the cloned bovine in the early gestation contributed to the fetus losses at early or later gestation of the cloned bovine. They also indicated that hydrallantois and enlarge placenta were found in the planceta of cloned bovine. Recently, the research on the embryonic gene expression of nuclear transfer indicated that the expression profiles of 3 of 6 genes in the early embryonic development stage were significantly different from the embryo produced through *in vitro* fertilization (Daniels *et al.*, 2000). As shown in the results of methylation level of specific DNA fragment, the specific DNA fragment was highly methylated in the somatic cell before the nuclear transfer process. The nuclear transfer embryo produced after nuclear transfer was highly methylated. On the other hand, the embryo produced by the specific DNA fragment through *in vitro* fertilization was slightly methylated. The results suggested that the recipient cell was unable to reprogram the genome and, consequently, the nuclear transfer embryo produced by the SCNT showed an inferior development ability. To the worst extent, the cloned calves became abnormal and the postnatal survivability dropped to a low level (Humpherys *et al.*, 2001; Kang *et al.*, 2001).

Although, we have generated one cloned transgenic goat, but all of five cloned calves were not harboring with the *aLA-hFVIII* transgene in this study. This was the same as results from other laboratory, which showed some animals generated by nuclear transfer from transfected cells failed to be transgenic. It is mostly due to the bystander effect of the transfected cells occurring in the selection process after foreign gene transfection, where transgenic cells, which express the antibiotic-resistance gene, provide protection to nearby nontransgenic cells either by secretion of the gene product into the medium or by direct cell-to-cell contact (Betthauser *et al.*, 2000; Bondioli *et al.*, 2001; Denning *et al.*, 2001; Echelard *et al.*, 2002; Lai *et al.*, 2002). Owing to the possible bystander effect in this study, the transfected donor cells might mixed and contain both transgenic and nontransgenic cells.

In conclusion, this study has confirmed that SCNT is a viable technique for bovine (Fig. 1) and goat (Fig. 1) cloning and produced one cloned-transgenic goat successfully. However, it is failed for obtaining a complete sequence of *hF VIII* transgene in the genome of the transgenic cloned calves. Further studies will be

required to gain those particular clones of donor cells harboring the intact transgene before being subjected to the nuclear transfer to ensure the desired transgenic offspring successfully cloned, and to evaluation the low cloning efficiency whether due to caused by incomplete reprogramming of the foreign nuclear materials or other factors.

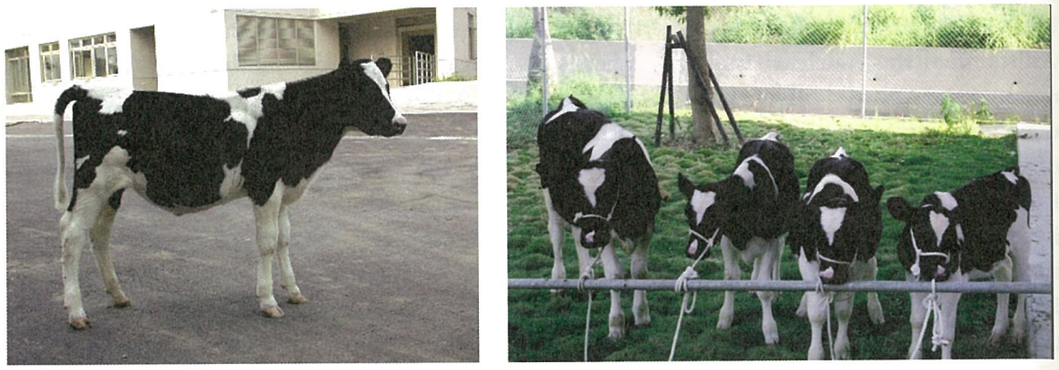

Fig. 1. Five cloned calves produced from an adult Holstein ear fibroblast cell of which transfected with *hFVIII* gene.

Fig. 2. A cloned-transgenic goat produced from adult Alpine ear fibroblast cell of which transfected with *hFVIII* gene.

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